

## Review article

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### THE CURRENT VIEW ON BIOLOGICAL POTENCY OF CATIONICALLY MODIFIED CHITOSAN

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Chitosan is biocompatible polymer obtained from chitin, the building component of the crustacean shells. In this paper we make an attempt to review the current state of knowledge on some biological effects of chitosan in comparison with those of cationically modified chitosan, N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC) that was recently synthesized by us by covalent attachment of glycidyltrimethylammonium chloride (GTMAC). Biological effects of HTCC and non-modified polymer are very similar. However, HTCC shows some unique beneficial properties which have not been found in its non-modified counterpart. One such example is that HTCC has the ability to bind heparin at physiological pH. HTCC having the degree of substitution almost 63.6% is easily absorbed within 1 hour after oral administration as found in C57BL/6j mice using FITC-labeled polymer. HTCC is distributed to lung, heart, and kidneys. HTCC stimulates and enhances blood platelet aggregation and decreases erythrocyte deformability (RBC). Moreover, HTCC seems to decrease both plasma total cholesterol level and LDL-cholesterol level in apoE-knockout mice fed with a diet containing HTCC. HTCC possibly down-regulates the HMG-CoAR mRNA level after 24 hour incubation with HepG2 cells *in vitro*.

**Key words:** *chitosan, cationically modified chitosan, quaternization, atherosclerosis, hypercholesterolemia, heparin, apoE-knockout mouse, platelet aggregation, red blood cells deformability, lipid metabolism*

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#### INTRODUCTION

Cationic polymers, both of natural and synthetic origin, have been intensively studied due to their immense therapeutic potential including delivery of gene which is probably their best known biomedical application (1). Synthetic polycations are synthesized often with desired molecular weight, they can be easily modified and do not show batch-to-batch variation, a typical disadvantage of natural polymers. The synthetic polycations, which are the most intensively studied in biomedical applications, especially in drug delivery, tissue engineering and gene delivery, are polyethyleneimines (PEIs) (2) poly(L-lysine) (PLL) (3), poly(amino-co-esters) (PAEs) (4, 5) and poly(dimethylaminoethylmethacrylates) (PDMAEMAs) (6, 7). However, they are often toxic, show poor hemocompatibility, and low biodegradability (8, 9).

On the other hand, the cationic polymers of natural origin are actually limited to proteins with chitosan being a unique polymer as the only natural cationic polysaccharide. Chitosan is a random copolymer of N-acetyl-D-glucosamine and D-glucosamine (10). In nature it occurs in small amounts in the cell walls of several genera of mushrooms *e.g.*, *Mucor*, *Rhizopus*, and *Absidia* (11, 12). However, on a synthetic scale it is obtained by deacetylation of chitin, the main component of the crustacean shells (13, 14) being regarding the validity of the importance second (after cellulose) substance in the biosphere. The process of chitin deacetylation may be carried out to different degree leading to

chitosans of various degrees of deacetylation (DD). DD and the molecular weight are two important characteristics of chitosan that determine its physicochemical properties, solubility in particular. Its structure, physicochemical properties and biological functions are determined by the presence of three types of functional reactive groups, *i.e.*, hydroxyl, amino, and acetamido groups. Chitosan is soluble only in acidic solutions. Its solubility is higher for chitosans with higher DDs and lower molecular weight (15). Chitosan has found many commercial applications in cosmetics (16), food industry (17), agriculture (18), environmental protection (19), wastewater management (20), and chemical industry (13).

#### UNMODIFIED CHITOSANS

Chitosan has many beneficial biomedical properties such as biocompatibility, biodegradability, and nontoxicity. Biological activity of chitosan is closely related to its solubility and thus to both molecular weight and DD. Decreasing molecular weight increases absorption of chitosan after oral administration in mice (21) and rats (22). When absorbed, low-molecular-weight chitosan was found to be distributed to various of tested organs, *i.e.*, liver, kidney, spleen, thymus, heart, and lung, and easily metabolized.

Chitosan decreases absorption of lipids in the intestine by binding bile acids and fatty acids (23). Therefore, the polysaccharide is a well-known dietary weight-reducing

supplement. Oral chitosan lowers plasma cholesterol (24, 25) without adverse effects. The mechanism of hypocholesterolemic action of chitosan is still unclear. However, it has been suggested that chitosan acts by inhibiting the absorption of cholesterol and bile acids (23). Due to stronger electrostatic attraction between this cationic polysaccharide and anionic substances like bile acids and fatty acids, chitosan with higher DD (about 90%) and molecular weight shows higher fat-binding capacity (12). On the other hand, its cholesterol lowering effect does not show a clear dependence on the above features (12). Its hypocholesterolemic potency seems to be related to inhibition of progression of atherosclerosis (26).

One of the most important properties of chitosan is its inherent antimicrobial effect, that was first recognized more than 40 years ago (27), against many Gram-positive and Gram-negative bacteria, yeasts, and fungi (28, 29).

Furthermore, chitosan is one of the most intensively studied and safe component of drug delivery systems (30) prepared in different forms, *e.g.* as a hydrogel (31), nanoparticles (32-35), microparticles (28), and films (36); and administered by various routes, including topical (33) *e.g.* general mucosal (37), nasal (31), intraocular (38) and oral. Chitosan-based oral drug delivery systems may be quite advanced and include oral insulin administration (32), colon drug delivery (39), gastroretentive floating drug systems (40), or gene delivery (41, 42).

Other pharmacological properties of chitosan are: antitumor (43), immuno-enhancing (44, 45), wound healing (46), and antidiabetic (47, 48). In biotechnology chitosan is employed in tissue engineering (49, 50) and as a cell culture support (51, 52). Chitosan reveals immuno-stimulating effect because of its impact on the body's immunological response to inflammation (45, 53). This polysaccharide increases production of interleukin 1 (IL-1), platelet-delivered growth factor, transforming growth factor  $\beta$ 1 and phagocytosis of macrophages. Chitosan enhances also phagocytosis of polymorphonuclear leukocytes (PMN) and production of leukotriene B4 and osteopontin. These effects are crucial for healing of large open wounds as found in animals. Chitosan polymers induce fibroblasts to release interleukin-8 (IL-8) involved in proliferation and migration of vascular endothelial cells and fibroblasts (45, 53). Chitosan when delivered intranasally enhances systemic and local immune responses to tetanus toxoid, influenza pertussis and diphtheria vaccines (45, 54-57). Similar effects were observed after oral gene-delivery with chitosan-DNA nanoparticles in animal studies (45, 58, 59). Another studies showed that chitosan might limit an immunosuppression (45, 59). It was observed that after chitosan subcutaneous implantation in dogs the number of white blood cells was increased (45, 60).

The poor solubility of chitosan in water significantly constrains its possible applications. These limitations can be at least partially overcome by its cationic modification with ammonium groups which are ionized irrespective of the pH value.

Biological activity of the cationically modified chitosan overlaps significantly with that of the native chitosan and includes antibacterial (60) and antifungal (61) properties, enhancing adsorption of hydrophilic drugs through mucous membranes (62-64) improving proliferation of human periodontal ligament cells (HPDLC) (65) and inhibition the proliferation of cancer cells (66).

However, some properties are unique to cationically-modified chitosan. Studies on one of these type of polymers, including our recently performed studies, are as follows:

#### *Antiheparin properties of cationically-modified chitosan*

As previously described by us cationic modification (quaternization) of chitosan was conducted by covalent attachment of glycidyltrimethylammonium chloride (GTMAC) (67).

Two polymers of N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride with degree of substitution (DS) 63.6% and 90.5%, referred to as HTCC1 and HTCC2 respectively, were obtained. The modified chitosans showed very good solubility in water at physiological pH. In PBS buffer at pH=7.4 the zeta potential of HTCC1 and HTCC2 was found to be  $13.28 \pm 1.21$  and  $24.2 \pm 0.83$  respectively, indicating that these polymers may have a tendency to the formation of polycation-polyanion complexes. Indeed, it was shown that HTCC polymers form complexes with heparin - a strongly anionic polysaccharide anticoagulant - accompanied by the inhibition of anticoagulative activity of both unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) (61). Moreover, heparin activity could not only be blocked by HTCC but also removed from the solution *in vitro* in the form of microspheres crosslinked with genipin, a naturally-occurring compound obtained from Gardenia fruit (68).

#### *Absorption and distribution of HTCC in mice after oral administration*

HTCC absorption we have studied in C57BL/6j mice after oral administration of this polymer previously labeled with fluorescein isothiocyanate (FITC) (unpublished data). High level of FITC fluorescence that has been found in serum 1 hour after oral administration indicates that HTCC is easily

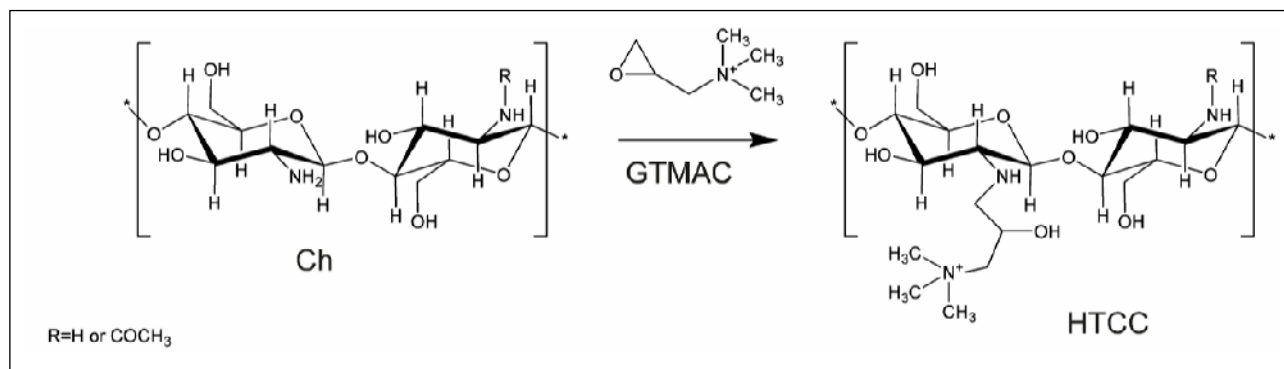


Fig. 1. Substitution reaction of chitosan with GTMAC. Ch, chitosan; GTMAC, glycidyltrimethylammonium chloride; HTCC, N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride.

absorbed from GI tract. HTCC was distributed mainly to lung and heart and particularly high HTCC concentration was found in kidney. Twenty four hours after oral administration HTCC was completely eliminated both from serum and the studied organs. However, there are studies indicating that some of unmodified high-molecular weight chitosan samples (HCS,  $M_w=760,000$  Da) would gradually precipitate at the lower intestine and would be hardly absorbed if their molecules were not degraded into soluble ones (21). In mice plasma level of HCS half an hour after oral administration was very low and this level was negligible later (1 hour time point or even at 4 hour time point after administration). In kidney the highest concentration of HCS was found after 2 hours and then at 4<sup>th</sup> hour was significantly decreased. A large quantity of HSC was observed in feces when HSC was used as dietary fiber supplements (21).

#### *HTCC stimulates and enhances platelet aggregation*

Chitosan with moderately high molecular weight ( $M_w=50,000$  Da) and high degree of deacetylation ( $DD>90\%$ ) enhances platelet aggregation and adhesion in rabbits, increases expression of platelet glycoprotein GPIIb/IIIa complex on platelet membrane and significantly increases platelet intracellular  $Ca^{2+}$  (69). We have found that HTCC with DS of 63.6% essentially stimulates human platelet aggregation *in vitro* and this phenomenon is dose-dependent (unpublished data). This phenomenon occurs in the absence of collagen and it is in contrast to platelet aggregation accompanying the bleeding from wounded vessels. The above results suggest that cationically modified chitosan may be an effective hemostatic agent but its hemostatic mechanism seems to be independent on the classical coagulation cascade (70). It also is well established that chitosan deprived cationic modification accelerate wound healing (71, 72). Unmodified chitosan inhibits

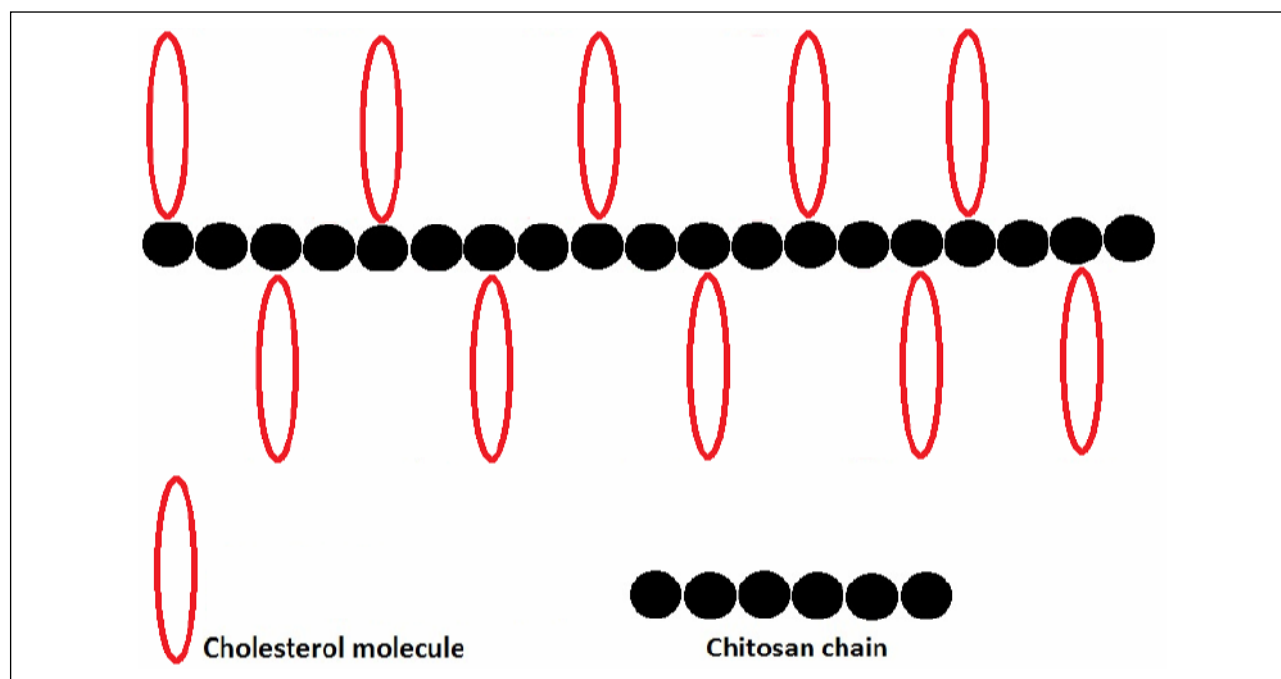
the diffuse capillary bleeding in brain tissue (73) and the lingual bleeding in heparinized rabbits (74).

#### *The effect on red blood cell deformability*

It is well established that atherosclerosis and hyperlipidemia are related to microcirculatory disturbances and they are accompanied by decrease of red blood cell (RBC) deformability. However, in rabbits with hypercholesterolemia induced by the hen's egg enriched diet the deformability of RBC was found to be increased (75). It was speculated that this could be a result of the changes in the mean corpuscular volume, viscosity of erythrocyte cytoplasm, and oxidative/antioxidative balance. We have found that HTCC with DS of 63.6% causes significant decrease of RBC deformability in human blood *in vitro* (unpublished data). Significant decrease of human RBC deformability was also found to be induced by chitooligosaccharide with  $M_w=50,000$  Da, however its potency was much lower than HTCC.

#### *The effect on lipid metabolism and hypercholesterolemia in the apolipoprotein E-deficient mouse model of atherosclerosis*

To study the effect of HTCC on lipid and cholesterol metabolism we have used the apolipoprotein E-knockout mice model of atherosclerosis (76, 77). The apoE-knockout homozygous mutant mice have total plasma cholesterol level five times higher than that of normal animals while triglyceride levels are 68% higher than in normal litter mates. The high density lipoprotein (HDL) cholesterol levels reach only 45% of the normal level. Gene-targeted apoE-knockout mouse model proved to be very useful in the development of the inflammatory theory of atherosclerosis (78). In addition, process of atherogenesis seems to be affected by a gender of ApoE(-/-) mice. This effect is connected with markedly elevated



*Fig. 2.* Molecular simulation of a chitosan-cholesterol complex obtained by Gaussian 98 software. The simulation displays the ability to create a specific sites of electrostatic bonds between chitosan chains and cholesterol molecules. It is believed that cholesterol do not cause great perturbation in the shape of the chitosan molecule. The interaction between chitosan and cholesterol was found between several charged sites of the chitosan molecules ( $-NH_3^+$ ) and the OH group of cholesterol. Distance between two sites of interaction of cholesterol with chitosan has been measured and the value is 9 Å.

production of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and reduced prostacyclin (prostaglandin I<sub>2</sub>, PGI<sub>2</sub>) production in female ApoE(-/-) mice (79). To restrain of atherosclerosis progress some compounds were studied in mice model successfully, *e.g.*, antileukotriene drugs (80), AVE 0991 - angiotensin(1-7) receptor agonist (81) and doxycycline (82). Chitooligosaccharides have angiotensin-I-converting enzyme (ACE) inhibitory activity (13, 83).

In apoE-deficient mice fed for 20 weeks on a diet containing 5% w/w of chitosan (DD=78%) blood cholesterol level was significantly decreased to 64% of that in the control animals (*i.e.* on a diet without chitosan). Examination of the area of aortic plaque showed a potent inhibition of atherogenesis in the whole aorta (about 42%) as well as in the aortic arch (50%) as compared to control mice (26). Moreover, we found that apoE-knockout mice fed for 16 weeks with a diet containing 200 mg of HTCC (with DS of 63.6%) per 1 kg of body weight, had total plasma cholesterol and LDL level lower by about 15% and 32%, respectively, as compared to the control (unpublished data). *Fig. 2* contains an explanation of the possible mechanism of binding of cholesterol to chitosan according to molecular simulation of complexes of chitosan-cholesterol obtained with the Gaussian 98 software (84).

It is supposed that cationically modified chitosan (HTCC) may form pseudomicelles with cholesterol. In these structures cholesterol is inside pseudomicelle and more hydrophobic fragments of chitosan (main hydrocarbon chain) are immersed in

hydrophobic centrum of cholesterol. Hydrophilic fragments of chitosan molecule through its electrostatic charges become oriented towards peripheries of pseudomicelles and this way the structures turn to more stabilized form. Stability of these microstructures in water environment possibly makes removal of cholesterol from the body much easier than in the form of agglutinated drops. Similar effect occurs in the case of fatty acids and their salts where pseudomicelles are additionally stabilized by electrostatic interactions between carboxyl groups of acids and cationic hydrophilic domains in chitosan molecule. In addition, it seems that these pseudomicellar structures may be stabilized by hydrogen bonds between number of OH groups present in chitosan and nucleophile atoms in cholesterol structure.

The effect of chitosan on the activity of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoAR) - a hepatic enzyme involved in metabolism of lipoproteins, has previously been studied in rats (85). In rats fed a chitosan-sterol diet (DD=92%) the activity of HMG-CoAR increased more than in those fed with a sterol diet but less than in rats fed with a normal diet. Further studies with rats fed with chitosan using semi-quantitative RT-PCR showed that chitosan could downregulate the HMG-CoAR mRNA level (13). We found that also HTCC significantly down-regulates the HMG-CoAR mRNA level after 24 hour incubation with HepG2 cells *in vitro* (real-time PCR results). This effect was not observed after incubation with

*Table 1.* Comparison of various biological characteristics of the native chitosan with HTCC.

Location	Native chitosan	HTCC
Blood	<ul style="list-style-type: none"> <li>- enhances platelet aggregation and adhesion in rabbits</li> <li>- decreases human red blood cells deformability</li> <li>- increases expression of platelet glycoprotein GPIIb/IIIa complex on platelet membrane</li> <li>- increases platelet intracellular Ca<sup>2+</sup></li> </ul>	<ul style="list-style-type: none"> <li>- forms complexes with heparin, a strongly anionic polysaccharide anticoagulant</li> <li>- stimulates and enhances platelet aggregation</li> <li>- decreases human erythrocyte deformability</li> <li>- hemostatic mechanism may be independent of the classical coagulation cascade</li> <li>- zeta potential of HTCC polymers may show a tendency to the formation of polycation-polyanion complexes</li> </ul>
Absorption and distribution (Pharmacokinetics)	<ul style="list-style-type: none"> <li>- reduced molecular weight increases absorption of chitosan after oral administration in mice and rats</li> <li>- when absorbed is distributed to liver, kidney, spleen, thymus, heart, and lung, then easily metabolized</li> </ul>	<ul style="list-style-type: none"> <li>- FITC-labeled is easily absorbed within 1h after oral administration in mice</li> <li>- distributed to lung, heart, and kidneys</li> </ul>
Bioavailability (Pharmacokinetics)	<ul style="list-style-type: none"> <li>- at pH 5 solubility is only infinitesimal so bioavailability is minimal</li> </ul>	<ul style="list-style-type: none"> <li>- higher bioavailability than unmodified chitosan due to its better solubility in physiological conditions</li> <li>- well soluble at blood pH, so may stay in blood for longer time and may affect the level of fatty acids and cholesterol</li> </ul>
Absorption in GI track	<ul style="list-style-type: none"> <li>- interacts strongly with cholesterol in the stomach leading to slowing of cholesterol absorption</li> </ul>	<ul style="list-style-type: none"> <li>- due to its solubility in wide range of pH it should probably be well absorbed not only from the stomach</li> </ul>
Lipid metabolism	<ul style="list-style-type: none"> <li>- lowers plasma cholesterol level after oral administration justifying the idea that it may inhibit progression of atherosclerosis</li> <li>- conception that it may inhibit progression of atherosclerosis</li> <li>- decreases absorption of lipids in the intestine by binding bile acids and fatty acids</li> <li>- due to stronger electrostatic attraction between its cationic part of molecule and anionic substances, like bile acids and fatty acids it shows higher fat-binding capacity. Cholesterol lowering effect does not seem to be dependent on this phenomenon</li> <li>- the interaction between chitosan and cholesterol was found to be located between a charged particles of the chitosan (-NH<sub>3</sub><sup>+</sup>) and the OH group of cholesterol</li> <li>- in rats fed with a chitosan/sterol diet the activity of HMG-CoAR is increased more than in those fed with a sterol diet but less than fed with a normal diet</li> <li>- chitosan administered orally may down-regulate the HMG-CoAR mRNA level in rats</li> </ul>	<ul style="list-style-type: none"> <li>- decreases both plasma total cholesterol level and LDL- cholesterol level in apoE-knockout mice when they fed with it in daily diet</li> <li>- it may form pseudomicelles with cholesterol. The stability of pseudomicelles in water environment makes cholesterol easier to be removed. Similar phenomenon occurs with fatty acids and their salts when pseudomicelles are stabilized by electrostatic interactions</li> <li>- down-regulates the HMG-CoAR mRNA level after 24 hour incubation with HepG2 cells <i>in vitro</i>. This effect is not observed after incubation with chitooligosaccharides deprived cationic modification</li> </ul>
Skin	<ul style="list-style-type: none"> <li>- accelerates wound healing</li> </ul>	?

chitoooligosaccharide ( $M_w=5,000$  Da) deprived cationic modification (unpublished data).

*Is cationically modified chitosan (HTCC) more efficient in regulating lipid metabolism than native chitosan?*

It is believed that HTCC has higher bioavailability than unmodified chitosan and this due to its better solubility in physiological conditions. Native chitosan at pH 5 is soluble only infinitesimally so its bioavailability is minimal. On the other hand, at pH in the stomach unmodified chitosan becomes soluble and in such conditions it may easily be absorbed to blood. But pH of blood is 7.4, so concentration of the chitosan in blood is expected to be rather low and then chitosan may be subject to rapid uptake by organs. Furthermore, beneficial function of chitosan is binding of fatty acids and cholesterol in blood. Unmodified chitosan interacts with cholesterol in the stomach so effectively that practically it doesn't let cholesterol to be absorbed from digestive tract. In contrast to unmodified chitosan, HTCC, due to its solubility at wide range of pH, has a big chance to be absorbed not only from the stomach. On the other hand, HTCC is well soluble at pH of blood, so it may stay in blood for longer time and therefore to influence the blood level of fatty acids and cholesterol very effectively. To sum up, HTCC having the characteristics of a chitosan it is just a more effective (Table 1).

In summary, HTCC is a well-absorbed cationic chitosan derivative that displays important biological activities including recently found effect on lipid metabolism. Some of its activities, e.g. binding of heparin or cholesterol lowering effect, appear to be quite unique. Although HTCC in principle mimics the actions of unmodified chitosan but it is doing this with clearly better effectiveness and this may prove useful from the therapeutic point of view.

**Acknowledgements:** Authors JS and RK acknowledge the financial support from the project Interdisciplinary PhD Studies "Molecular sciences for medicine" (co-financed by the European Social Fund within the Human Capital Operational Programme). Co-authors MN and KS gratefully acknowledge financial support of the Foundation for Polish Science TEAM Programme (PolyMed, TEAM/2008-2/6) co-financed by the European Regional Development Fund.

Conflict of interests: None declared.

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Received: November 27, 2013

Accepted: April 2, 2014

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